MBMG
Metabarcoding & Metagenomics

Metabarcoding and Metagenomics 2: 1–11
DOI 10.3897/mbmg.2.24556

Methods

Mu-DNA: a modular universal DNA extraction method adaptable for a wide range of sample types

Graham S. Sellers[1], **Cristina Di Muri**[1], **Africa Gómez**[1], **Bernd Hänfling**[1]

1 *School of Environmental Sciences, University of Hull, Hull, United Kingdom*

Corresponding author: *Graham S. Sellers* (graham.s.sellers@gmail.com)

Academic editor: *Per Sundberg* | **Received** 20 March 2018 | **Accepted** 4 June 2018 | **Published** 5 July 2018

Abstract

Efficient DNA extraction is fundamental to molecular studies. However, commercial kits are expensive when a large number of samples need to be processed. Here we present a simple, modular and adaptable DNA extraction 'toolkit' for the isolation of high purity DNA from multiple sample types (modular universal DNA extraction method or Mu-DNA). We compare the performance of our method to that of widely used commercial kits across a range of soil, stool, tissue and water samples. Mu-DNA produced DNA extractions of similar or higher yield and purity to that of the commercial kits. As a proof of principle, we carried out replicate fish metabarcoding of aquatic eDNA extractions, which confirmed that the species detection efficiency of our method is similar to that of the most frequently used commercial kit. Our results demonstrate the reliability of Mu-DNA along with its modular adaptability to challenging sample types and sample collection methods. Mu-DNA can substantially reduce the costs and increase the scope of experiments in molecular studies.

Key Words

modular adaptability, spin column, high-throughput, cost effective, metabarcoding, metagenomics, eDNA

Introduction

Extraction of double stranded DNA (dsDNA) from samples is essential for molecular studies. However, the inevitable co-extraction of contaminants, in particular humic substances, phenolic compounds and proteins, inhibit polymerase chain reaction (PCR) and other downstream applications (Tebbe and Vahjen 1993, Wilson 1997). Numerous published methods and commercial kits are available for the extraction of high purity DNA suitable for downstream applications. Many published methods are complex and designed for expert use, while commercial kits are readily accessible for those with little experience.

The DNeasy extraction kits (Qiagen) are simple, accessible and widely used. Although designed for specific sample types, many studies have adapted their use across sample types. DNeasy PowerSoil, or aspects thereof, has been used for stomach, gut or faecal analysis of invertebrates (Knapp et al. 2010, O'Rorke et al. 2015), fish (Koinari et al. 2013, Bolnick et al. 2014), reptiles (Lau et al. 2013, Colston et al. 2015), birds (Vo and Jedlicka 2014, Lewis et al. 2016), mammals (Parfrey et al. 2014, Ishaq and Wright 2014) and, in particular, the Human Microbiome Project (Aagaard et al. 2013). DNeasy Blood and Tissue has been used for studies of environmental DNA (eDNA) from water samples (Rees et al. 2014, Spens et al. 2016, Niemiller et al. 2017). Although widely used, commercial kits are expensive and separate kits can be required for different sample types. DNA extraction, using commercial kits, is therefore a significant cost factor which limits the scope of experiments in molecular studies and increases the costs of genetic biodiversity monitoring.

Here we present a modular universal DNA extraction method (Mu-DNA) to address the issue of the many kits, protocols and expense, for low cost application across multiple sample types. Mu-DNA is a cost-effective and adaptable high-throughput spin column-based protocol for the extraction of high purity DNA from multiple sample types.

Copyright *Graham S. Sellers et al.* This is an open access article distributed under the terms of the Creative Commons Attribution License (CC BY 4.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

PENSOFT

This is not a *de novo* method but an accessible combination of multiple aspects from recent and classical procedures for DNA extraction and purification. The method is based around easy-to-prepare reagents with an absolute minimum of pH adjustment required. As a modular approach, it uses reagent combinations dependent upon the sample type; soil, tissue or water. The method consists of five simple steps, all interchangeable between protocols, based around spin column DNA purification. We compared the performance of our Mu-DNA method, in particular, dsDNA yield, purity, downstream inhibition and extracted DNA molecular weight to that of the widely used commercial extraction kits: DNeasy PowerSoil, DNeasy Blood and Tissue and DNeasy PowerWater (Qiagen). Finally, we demonstrate the performance of the method in a comparative metabarcoding of fish community composition from oligotrophic lake water DNA extractions.

Methods

Solutions and reagents

We provide optimised Mu-DNA protocols for soil, tissue and water samples (Detailed protocols can be found at: https://doi.org/10.17504/protocols.io.qn9dvh6). Each protocol consists of five stages for DNA extraction: lysis, inhibitor removal, silica binding, wash and elution (Figure 1). Mu-DNA uses a lysis buffer modified from Brolaski et al. (2008). The buffer incorporates guanidine thiocyanate to denature proteins (Pitcher et al. 1989), trisodium phosphate to release adsorbed DNA (Ogram et al. 1987) and ethylenediaminetetraacetic acid (EDTA) to reduce DNA oxidation from metal ions (Lloyd and Phillips 1999). A sodium dodecyl sulphate (SDS) solution is added to the lysis buffer in all protocols to disrupt lipid membranes and degrade proteins. The presence of both EDTA and SDS at the lysis stage inhibits nuclease activity (Williams et al. 1980), greatly reducing the degradation of DNA. For soil extractions, the SDS additive includes aluminium ammonium sulphate to reduce humic substances (Braid et al. 2003). For soil and water filter extractions, bead milling is performed for unbiased high yield DNA liberation (Robe et al. 2003). Tissue extractions have a Proteinase K incubation period for enzymatic lysis and protein digestion. Soil and water lysates are purified with a contaminant and inhibitor removal solution. This contains ammonium acetate to precipitate proteins (Crouse and Amorese 1987), aluminium ammonium sulphate (Braid et al. 2003) and calcium chloride (Wechter et al. 2003, Singh et al. 2014) to remove contaminants and inhibitors, in particular remaining humic substances and fine sediment particles. DNA is subsequently bound to a spin column silica membrane under chaotropic conditions with guanidine hydrochloride (Davis et al. 1997). Ethanol washes then remove remaining contaminants prior to elution.

The stages of Mu-DNA are designed to be modular and interchangeable between protocols to facilitate optimisation of extraction methods for a given sample type. For example, a bead milling or inhibitor removal stage can be incorporated in a tissue extraction protocol and a tissue wash stage added to a soil or water extraction protocol. All processes are scalable based upon initial sample amount or transferred supernatant volumes.

Comparison of DNA yield and quality

To determine the performance of Mu-DNA, isolated DNA yield and purity were compared to that from the relevant commercial kits across soil, stool, tissue and water samples (Table 1). The molecular weight of extracted DNA from soil, tissue and water samples was compared between respective methods. Three to five biological replicates were performed per extraction method for each sample.

Sample selection

For each sample type, three different samples (A, B and C) were selected for comparison (Table 1). Sample A represented a commonly encountered sample of its type whereas B and C were representative of more challenging samples.

Sample preparation

Soil samples were collected from three soil types: A (garden soil; high organic content), B (ephemeral pool sediment; high clay content) and C (diesel polluted soil; high contaminant levels). All samples were loosely mixed at collection. In sterile laboratory conditions, 5 g of each sample was put through a 2 mm mesh sieve to remove large particulate debris before being thoroughly homogenised with a pestle and mortar. The homogenate was separated into multiple 0.25 g (wet weight) subsamples and stored at -20 °C until required for extraction.

Stool samples were collected from three species with different diets: A (European hedgehog, *Erinaceus europaeus*; omnivore), B (Greylag goose, *Anser anser*; grazer) and C (Otter, *Lutra lutra*; carnivore, high number of volatile organic compounds). In sterile laboratory conditions, each sample was thoroughly homogenised with a pestle and mortar. The homogenate was separated into multiple 0.25 g (wet weight) subsamples and stored at -20 °C until required for extraction.

Tissue samples were taken from ethanol preserved specimens of three species: A (Cichlid, *Nimbochromis livingstonii*; muscle tissue), B (Woodlouse, *Oniscus asellus*; high chitin content) and C (Earthworm, *Lumbricus terrestris*; mucus rich with soil gut contents). Multiple 25 mg (dry weight) subsamples of specimens were removed and stored at -20 °C until required for extraction.

Three water samples types were collected: A (shallow eutrophic lake; high sediment load and faecal matter), B (ephemeral pool mesocosm; turbid, high algal content) and C (deep oligotrophic lake; low particulate matter). After collection, samples were transported on ice and stored at 4 °C until filtered. Filtering took place less than

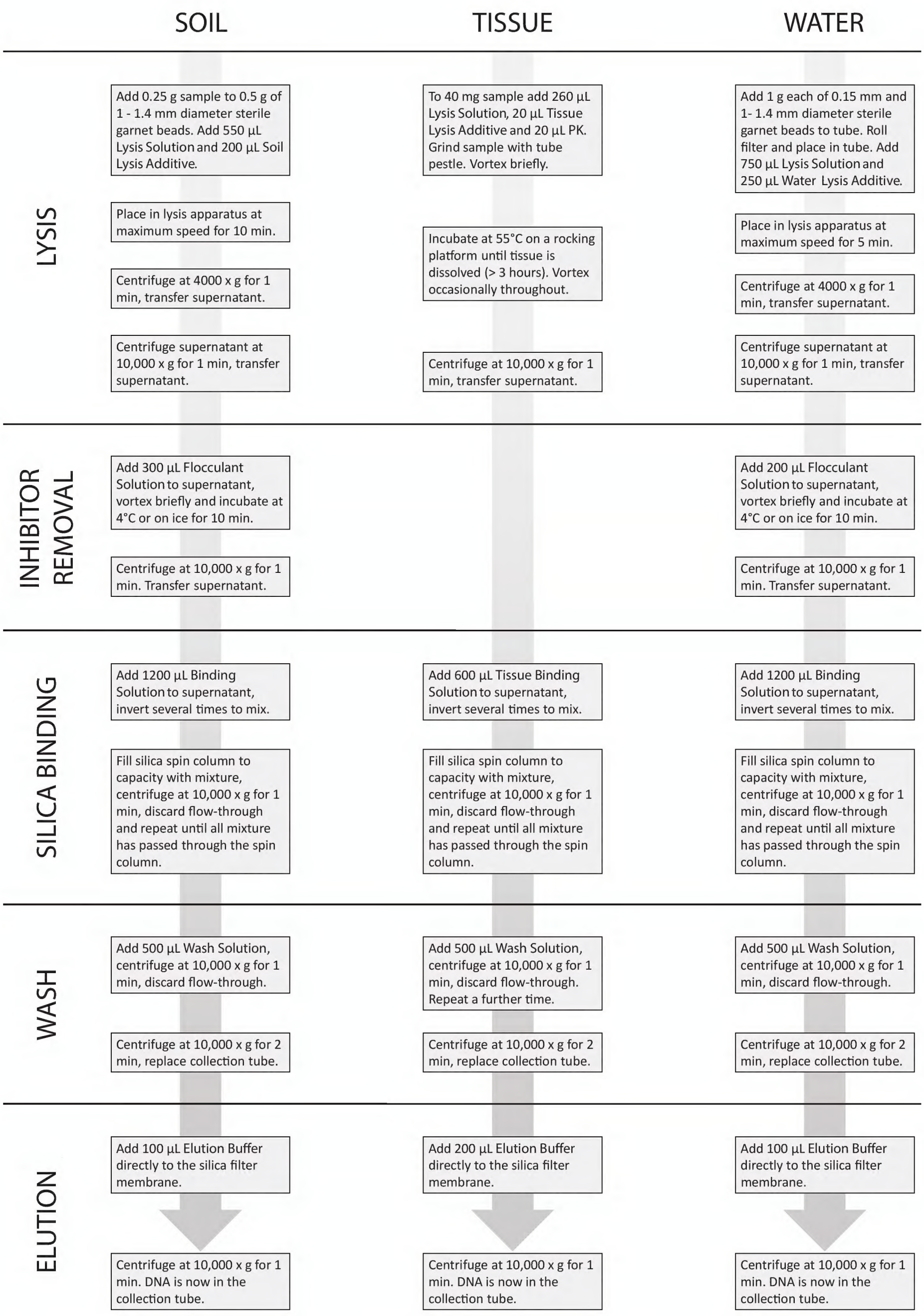
SOIL
TISSUE
WATER
LYSIS
Add 0.25 g sample to 0.5 g of 1 - 1.4 mm diameter sterile garnet beads. Add 550 µL Lysis Solution and 200 µL Soil Lysis Additive.
To 40 mg sample add 260 µL Lysis Solution, 20 µL Tissue Lysis Additive and 20 µL PK. Grind sample with tube pestle. Vortex briefly.
Add 1 g each of 0.15 mm and 1- 1.4 mm diameter sterile garnet beads to tube. Roll filter and place in tube. Add 750 µL Lysis Solution and 250 µL Water Lysis Additive.
Place in lysis apparatus at maximum speed for 10 min.
Incubate at 55°C on a rocking platform until tissue is dissolved (> 3 hours). Vortex occasionally throughout.
Place in lysis apparatus at maximum speed for 5 min.
Centrifuge at 4000 x g for 1 min, transfer supernatant.
Centrifuge at 4000 x g for 1 min, transfer supernatant.
Centrifuge supernatant at 10,000 x g for 1 min, transfer supernatant.
Centrifuge at 10,000 x g for 1 min, transfer supernatant.
Centrifuge supernatant at 10,000 x g for 1 min, transfer supernatant.
INHIBITOR REMOVAL
Add 300 µL Flocculant Solution to supernatant, vortex briefly and incubate at 4°C or on ice for 10 min.
Add 200 µL Flocculant Solution to supernatant, vortex briefly and incubate at 4°C or on ice for 10 min.
Centrifuge at 10,000 x g for 1 min. Transfer supernatant.
Centrifuge at 10,000 x g for 1 min. Transfer supernatant.
SILICA BINDING
Add 1200 µL Binding Solution to supernatant, invert several times to mix.
Add 600 µL Tissue Binding Solution to supernatant, invert several times to mix.
Add 1200 µL Binding Solution to supernatant, invert several times to mix.
Fill silica spin column to capacity with mixture, centrifuge at 10,000 x g for 1 min, discard flow-through and repeat until all mixture has passed through the spin column.
Fill silica spin column to capacity with mixture, centrifuge at 10,000 x g for 1 min, discard flow-through and repeat until all mixture has passed through the spin column.
Fill silica spin column to capacity with mixture, centrifuge at 10,000 x g for 1 min, discard flow-through and repeat until all mixture has passed through the spin column.
WASH
Add 500 µL Wash Solution, centrifuge at 10,000 x g for 1 min, discard flow-through.
Add 500 µL Wash Solution, centrifuge at 10,000 x g for 1 min, discard flow-through. Repeat a further time.
Add 500 µL Wash Solution, centrifuge at 10,000 x g for 1 min, discard flow-through.
Centrifuge at 10,000 x g for 2 min, replace collection tube.
Centrifuge at 10,000 x g for 2 min, replace collection tube.
Centrifuge at 10,000 x g for 2 min, replace collection tube.
ELUTION
Add 100 µL Elution Buffer directly to the silica filter membrane.
Add 200 µL Elution Buffer directly to the silica filter membrane.
Add 100 µL Elution Buffer directly to the silica filter membrane.
Centrifuge at 10,000 x g for 1 min. DNA is now in the collection tube.
Centrifuge at 10,000 x g for 1 min. DNA is now in the collection tube.
Centrifuge at 10,000 x g for 1 min. DNA is now in the collection tube.

Figure 1. Simplified Mu-DNA extraction protocols for soil, tissue and water samples. All extractions use stock and working solutions and are divided into five interchangeable stages: lysis, inhibitor removal, silica binding, wash and elution.

Table 1. Samples used for comparison of methods in this study. Shown are the amounts of each sample processed per extraction method: either Mu-DNA or the relevant commercial kit (Qiagen DNeasy).

Sample	Description	Area sampled	Sample amount	Extraction methods	Lysis apparatus	Replicates
Soil A	Garden soil	Topsoil – surface 5 cm	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Soil B	Ephemeral pool sediment	Topsoil – surface 5 cm	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Soil C	Diesel polluted soil	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	3
Stool A	*Erinaceus europaeus*	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Stool B	*Anser anser*	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Stool C	*Lutra lutra*	All available	0.25 g	PowerSoil Mu-DNA: Soil	Tissuelyser II	5
Tissue A	*Nimbochromis livingstonii*	Flank muscle	25 mg	Blood and Tissue Mu-DNA: Tissue	NA	5
Tissue B	*Oniscus asellus*	Lateral half	25 mg	Blood and Tissue Mu-DNA: Tissue	NA	3
Tissue C	*Lumbricus terrestris*	Central segments	25 mg	Blood and Tissue Mu-DNA: Tissue	NA	3
Water A	Shallow eutrophic lake	Shoreline surface	150 mL	PowerWater Mu-DNA: Water	Tissuelyser II	5
Water B	Ephemeral pool mesocosm	Surface	50 mL	PowerWater Mu-DNA: Water	Vortex Adapter	3
Water C	Deep oligotrophic lake	Shoreline surface	1 L	PowerWater Mu-DNA: Water	Vortex Adapter	5

16 hours after collection in sterile laboratory conditions. Each water sample was thoroughly mixed by pouring and then split into two subsamples of equal volume. Subsamples were vacuum-filtered through sterile 47 mm diameter 0.45 µm Whatman cellulose nitrate membrane filters (GE Healthcare), labelled and stored at -20 °C until required for extraction.

DNA extraction

DNA extractions of replicate samples followed the protocol of Mu-DNA for the sample type or the relevant DNeasy kit (Table 1). Protocols were modified as follows: Lysis and DNA purification for all protocols were carried out using identical lysis apparatus and spin columns. Soil and stool samples were lysed in 2 ml microcentrifuge tubes (Starlab) on a TissueLyser II (Qiagen) at 30 Hz for 10 minutes. Water samples were lysed in 7 ml Bijou tubes (Sigma-Aldrich) on either a TissueLyser II at 30 Hz for five minutes or Vortex Genie (Scientific Industries) with Vortex Adapter (MoBio) at maximum speed for five minutes. The DNeasy PowerSoil and DNeasy PowerWater Bead Tube contents were transferred to the new tube type prior to lysis. Tissue samples were lysed overnight for identical time periods and incubated at the temperatures specified per protocol. Where required, all available supernatant was transferred and reagent volumes were adjusted accordingly. EZ-10 DNA Mini Spin Columns (NBS Biologicals) were used for DNA purification in all protocols. Elution buffers used in each protocol were added to spin column membranes and left to incubate at room temperature for one minute before final collection. A single elution of the specified volume was performed for each protocol.

Extracted DNA yield, purity and downstream inhibition

dsDNA yield from all extractions was measured with a Qubit 3.0 fluorometer high-sensitivity (HS) dsDNA assay (Invitrogen). Isolated DNA purity was measured with a Nanodrop 1000 spectrophotometer (Thermo Fisher Scientific) recording A_{260}/A_{280} and A_{260}/A_{230} ratios for all extractions (see Olson and Morrow 2012). To test for the presence of any inhibiting factors in downstream applications, PCRs were run on all extractions. No PCR additives, such as BSA, were used to enhance PCR amplification. DNA extractions were amplified using the broad range DNA barcoding primers LCO1490 and HCO2198 (Folmer et al. 1994). PCRs were 25 µl final reaction volumes composed of 1 µl template DNA, 12.5 µl MyTaq Red Mix (Bioline), 9.5 µl ddH_2O and 1 µl of each 10 µM primer. All PCRs were performed on Veriti 96-Well Thermal Cyclers (Applied Biosystems) under the cycling conditions: 180 s at 94 °C, 37 × (30 s at 94 °C, 60 s at 52 °C, 90 s at 72 °C), 600 s at 72 °C, 600 s at 4 °C. PCR products were visualised on 1.5% agarose gels. All amplifications were given a PCR index score in comparison to a strong positive as follows: no amplification (0), weak amplification (1), moderate amplification (2) and strong amplification (3). Inhibition was considered present in an extraction if its index

was '0'. To determine the extent of inhibition exhibited in samples, those with a PCR index of '0' underwent further PCRs at 1:10, 1:100 and 1:1000 dilutions.

Extracted DNA integrity and molecular weight

To assess the integrity and molecular weight of DNA from the Mu-DNA protocols for soil, tissue and water, extractions were compared to those of their commercial counterparts. The highest yielding sample extractions per method were chosen from the highest yielding sample type. Selected extractions (5 µl) were visualised on a 0.5% agarose gel against a GeneRuler 1 kb Plus DNA Ladder (Thermo Fisher Scientific).

Adaptability of Mu-DNA

To demonstrate its adaptability, Mu-DNA was optimised for samples where inhibition (PCR indices of '0') was evident. Optimised protocols were then compared to the relevant commercial kit in fresh extractions from sample remnants.

Fish metabarcoding of lake water DNA extractions

Sample collection and preparation

A minimum of 2 l of water was collected from 13 shore sample sites around Windermere (Lake District, Cumbria, UK). Samples were transported on ice. Under sterile laboratory conditions, samples were thoroughly mixed by pouring and split into paired 1 l subsamples to be filtered. Filtering took place in less than 16 hours and filters were stored as above. DNA extractions followed the protocol of Mu-DNA: Water described above or DNeasy PowerWater. Identical lysis and purification conditions for both protocols were maintained: all filters were lysed in DNeasy PowerWater Bead Tubes and MB Spin Columns (Qiagen) were used for purification of all subsamples. Lysis was performed on a Vortex Genie (Scientific Industries) with Vortex Adapter (MoBio) at maximum speed for five minutes.

Library preparation

A double-indexed library was prepared following a 2-step PCR based protocol (Kitson et al. 2018) using primers for the vertebrate 12S mitochondrial gene region (Riaz et al. 2011, Kelly et al. 2014). In short, an initial PCR reaction amplified the target region using individually indexed 12S primers for each sample. To minimise PCR and sequencing bias, three sets of three PCR replicates per sample were performed to create three technical replicates with individual indices. Collection blanks were included in PCRs along with positive and negative controls. Replicate PCR products were pooled and purified using double-size selection with Mag-Bind RNXPure Plus beads (Omega Bio-tek) to remove nonspecific products and primer dimers. Final library concentration was assessed via qPCR assay using the NEBNext library quantification kit (New England Biolabs) and diluted as required to a final concentration of 4 nM. The final library was run at 15 pM concentration with 10% PhiX on an Illumina MiSeq using 600 bp V3 chemistry. A detailed protocol can be found in Supplementary file 1.

Bioinformatics and data analyses

metaBEAT, a custom bioinformatics pipeline (https://github.com/HullUni-bioinformatics/metaBEAT), was used to process sequencing outputs. The workflow consisted of the following steps: (i) demultiplexing; (ii) trimming, merging and quality filtering; (iii) chimera detection; (iv) clustering; (v) taxonomic assignment against a curated database. A low-frequency noise threshold approach was used to remove potential false positives from the metaBEAT data (Hänfling et al. 2016), only records exceeding a minimum proportion (0.001) of read counts in a sample were accepted as "true" positive records. Remaining reads were converted to relative species abundance (%) of assigned reads.

Statistical analysis

All statistical analysis was performed in R 3.2.5 (R Core Team 2016) using the VEGAN package (Oksanen et al. 2017). DNA yield and purity measures for extractions were compared with a linear model using planned contrasts between methods per sample. Metabarcoding of lake water DNA extractions were analysed using an analysis of similarity (ANOSIM) of relative species abundance across all replicates between methods. Non-metric multidimensional scaling (NMDS) ordination was used to visualise differences in extraction methods across all replicates grouped by site and extraction.

Costing of extraction methods

A cost per extraction was calculated for Mu-DNA: Soil, Tissue and Water. Costs per extraction were compared to those of DNeasy PowerSoil, DNeasy Blood and Tissue and DNeasy PowerWater, respectively. All costs used for comparisons were based on undiscounted list prices (GBP excluding VAT and shipping) for chemicals, plastics (excluding pipette tips) and Qiagen kits.

Data accessibility

Raw data and scripts are available on Open Science Framework (https://doi.org/10.17605/osf.io/vrb4a). Sequencing data are available from NCBI Sequence Read Archive (Bioproject: PRJNA473636, SRA accession numbers: SRR7234627–SRR7234708).

Results and discussion

Extracted DNA yield, purity and downstream inhibition

Our Mu-DNA method exhibited similar, if not significantly higher, dsDNA yields than the DNeasy kits for most extractions (Figure 2). The DNeasy kit achieved sig-

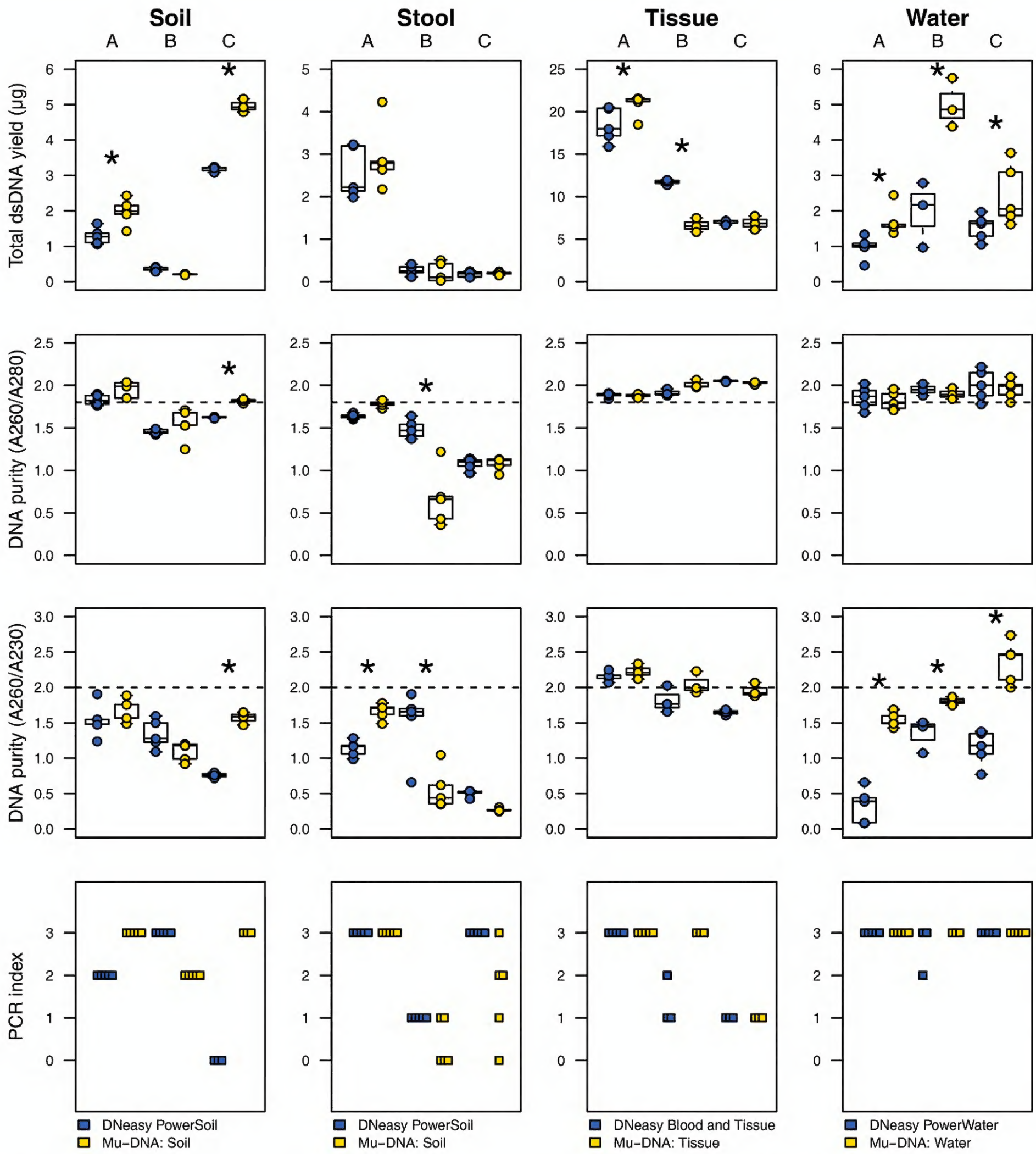

Figure 2. Isolated dsDNA yield, purity and PCR index of samples used in the comparison of methods. Total dsDNA yield, A_{260}/A_{280}, A_{260}/A_{230} ratios and PCR indices are shown for soil, stool, tissue and water samples per method. Horizontal dashed lines indicate ideal measures of A_{260}/A_{280} and A_{260}/A_{230} ratios for pure DNA. Asterisks indicate significant differences between methods (planned contrast linear model, $p<0.05$).

nificantly higher dsDNA yields than Mu-DNA only for Tissue B (woodlouse). A_{260}/A_{230} ratios for all extractions were similar or significantly higher for Mu-DNA except Stool B, which was significantly lower than the DNeasy kit. Soil B had higher A_{260}/A_{230} ratios from the DNeasy kit whereas Soil C had a significantly higher value from Mu-DNA. Stool A had higher A_{260}/A_{230} values from Mu-DNA whereas Stools B and C had higher values from the DNeasy kit. All A_{260}/A_{230} purity measures for tissue extractions were similar. All water sample extractions had higher A_{260}/A_{230} measures from Mu-DNA. PCR inhibition (PCR index of '0' in some or all extractions) was only detected in commercial kit extractions for Soil C and Mu-DNA extractions for Stools B and C. All other samples exhibited complete PCR success from both methods. Mu-DNA had a higher PCR index than the DNeasy kit for Tis-

sue B despite having lower dsDNA yield. PCR inhibition was overcome by extraction dilution (1:10) for DNeasy kit extractions for Soil C and Mu-DNA extractions for Stool C. However, Mu-DNA extractions for Stool B failed to amplify across all extractions at any dilution tested, indicative of high level inhibition.

The DNeasy kits reliably extracted inhibition-free DNA from all sample types except Soil C (diesel polluted soil). Compared to this baseline of extraction success, our Mu-DNA protocols, with the exception of two samples (Stools B and C), performed similarly. Therefore the three basic Mu-DNA protocols we provide for soil, tissue and water are highly suitable for many sample types. Our unmodified protocols successfully extracted inhibition-free DNA from 10 out of 12 of the samples tested in this study. Modification of our protocols for the more challenging samples is described later (see Adaptability of Mu-DNA).

We used A_{260}/A_{280} and A_{260}/A_{230} UV absorbance measures via spectrophotometry to determine the quality of DNA extractions as suggested by Olson and Morrow (2012). The ideal measures for pure DNA are shown in Figure 2, yet in some cases, they are exceeded. These measures can be influenced by many aspects, such as invertebrate chitin (Athanasio et al. 2016) and RNA. Spectrophotometry of extracted DNA can be affected by the presence of co-extracted RNA by inflating A_{260} values, therefore the ratios used for purity evaluations are skewed upwards. In our study, we refrained from the use of RNase so as to give a true representation of the method in an unmodified state. Should RNA-free DNA be required for any sample type, we suggest an RNase A treatment for a short incubation period (<1 hour) post-lysis. As purity measures can be affected by many factors, extracted DNA quantity and quality can therefore only reliably be ascertained by a combination of high-sensitivity dsDNA assays, gel electrophoresis visualisation of extracted DNA and intensity of PCR amplification success.

Extracted DNA integrity and molecular weight

The highest yielding extractions per method (Qiagen DNeasy or Mu-DNA) from Soil C, Tissue A and Water B were selected for DNA integrity and molecular weight visualisation (Figure 3). All extractions had a molecular weight of approximately 10 kbp or higher. Similar integrity of extracted DNA was observed in the soil and water samples, however DNeasy Blood and Tissue had poor integrity compared to Mu-DNA: Tissue.

High molecular weight DNA extraction is desirable for many next generation sequencing (NGS) studies. It also allows for long range PCR amplification of whole mitochondrial genomes from eDNA samples (Deiner et al. 2017). Bead milling lysis has been shown to cause shearing of nucleic acids, resulting in low molecular weight of extracted DNA (Bürgmann et al. 2001). Our method yielded DNA of ≥10 kbp (Figure 3) in bead milled extractions but shearing is still present, evident in an extended smear. However, Mu-DNA protocols exhibited increased concentrations of

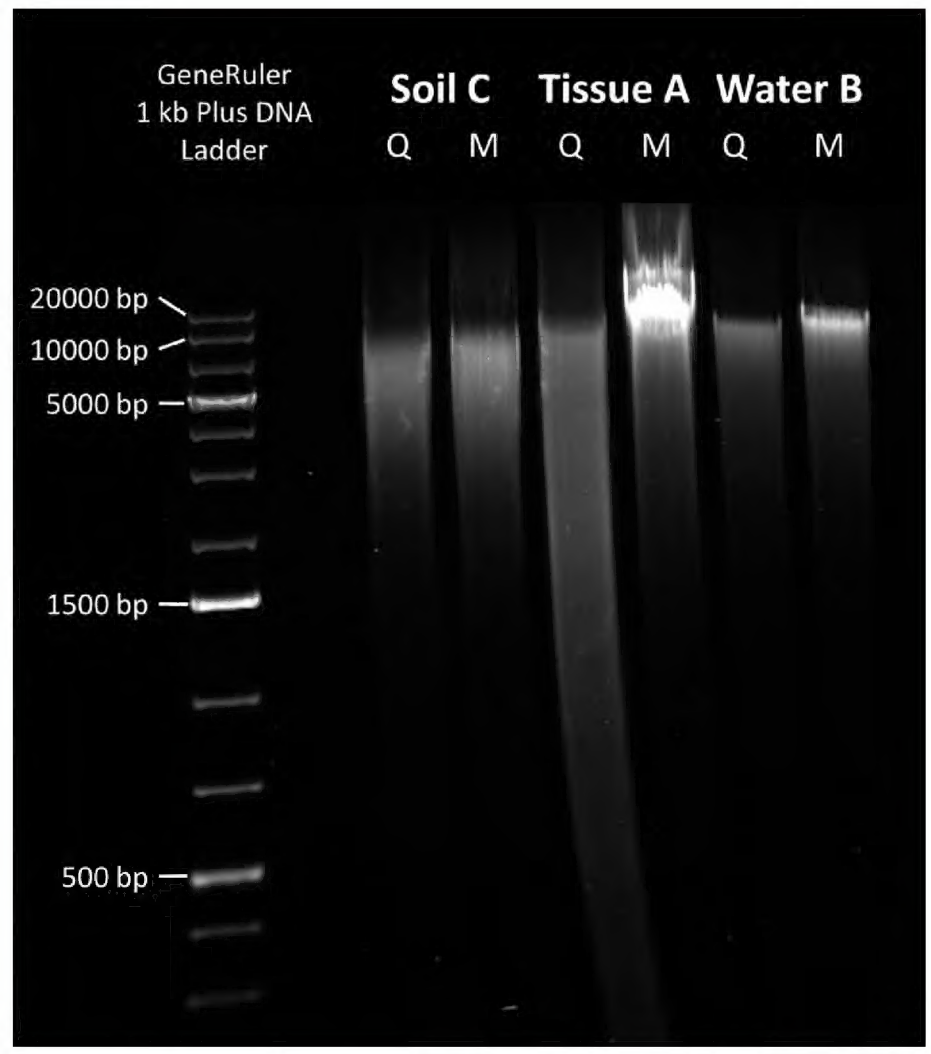

Figure 3. Integrity and molecular weight of soil, tissue and water sample extractions from the methods compared in this study. Shown are the highest yielding extractions per method from Soil C, Tissue A and Water B. Extractions are indicated by the relevant method for sample type; DNeasy (Q) or Mu-DNA (M).

higher molecular weight DNA than their commercial counterparts. Reducing bead milling times or enzyme digestion temperatures are both possible with Mu-DNA to reduce DNA shearing depending upon user end requirements. Additional measures can be taken to reduce the effects of physical and enzymatic shearing of DNA during sample preparation, extraction and even handling (see Klingstrom et al. 2018), yet these could become time-consuming for very large sample numbers.

Adaptability of Mu-DNA

PCR inhibition was present in DNA extractions of two samples for the Mu-DNA protocol: Stools B and C. The modular aspect of the Mu-DNA method was employed to optimise extractions for each of these samples to achieve complete initial PCR success. For Stool B, a tissue lysis stage that incorporated bead milling was used. A 0.25 g sample was added to 0.5 g of 1 – 1.4 mm garnet beads. A 2.5 x volume tissue lysis mixture was added. Soil protocol bead milling was performed followed by overnight tissue protocol incubation. The extraction then followed the soil protocol with a tissue protocol wash stage. For Stool C, the soil protocol was modified with a tissue protocol wash stage. These modifications improved DNA purity for both sample types with successful PCR amplification (Figure 4).

The modular adaptability of Mu-DNA allows for its application across different sample types or integration

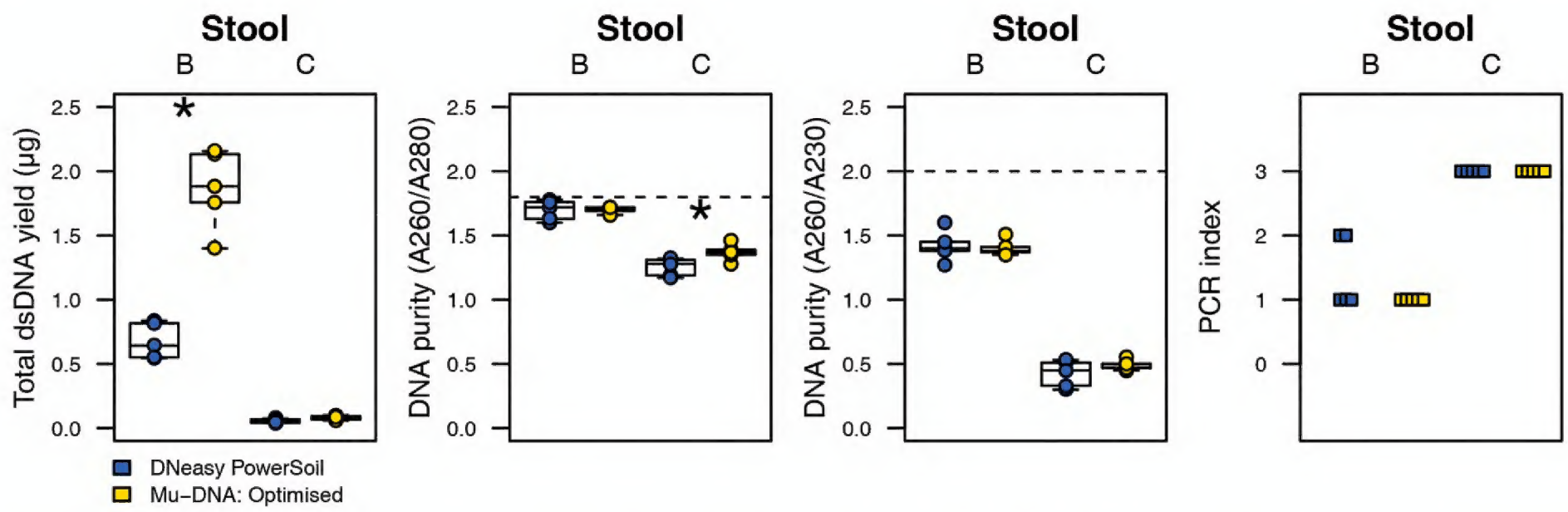

Figure 4. Optimised Mu-DNA protocols for stool samples that previously failed to achieve inhibition-free DNA. Optimised protocols are compared to DNeasy PowerSoil. Total dsDNA yield, A_{260}/A_{280}, A_{260}/A_{230} ratios and PCR indices are shown for stool samples B and C. Horizontal dashed lines indicate ideal measures of A_{260}/A_{280} and A_{260}/A_{230} ratios for pure DNA. Asterisks indicate significant differences between methods (planned contrast linear model, $p<0.05$).

into existing protocols. For example, Spens et al. (2016) made use of DNeasy Blood and Tissue in their protocols for water filters, including Sterivex filters. We adapted and tested these protocols (data not shown) and found them to be easily changed to use a Mu-DNA: Tissue/Water protocol. For this, we recommend beginning with a tissue protocol lysis, adjusting the volumes as required, then following the water protocol from inhibitor removal through to elution. In this way, contaminants are greatly reduced and there is no need for extra purification of extractions (unlike with Niemiller et al. 2017). However we found that neither DNeasy Blood and Tissue nor the adapted Mu-DNA: Tissue/Water protocol could achieve inhibition-free DNA from turbid, algal rich waters (Water B) as effectively as DNeasy PowerWater or Mu-DNA: Water (data not shown). Solid phase reversible immobilisation (SPRI) DNA purification, based on Rohland and Reich (2012), can achieve higher DNA yield and purity than spin column based protocols (Vo and Jedlicka 2014). The Mu-DNA method can be easily converted to SPRI purification by replacing the silica binding step with an SPRI protocol. However Vo and Jedlicka (2014) found SPRI to only have improved performance with less contaminated samples, such as avian oral and cloacal swab extractions. SPRI DNA purification is therefore best reserved for relatively clean environmental sample types, in particular clear lake and stream waters or tissue samples (see Mayjonade et al. 2016).

Our modular approach to DNA extractions is not a new concept. Lever et al. (2015) developed a modular extraction method for multiple environmental samples. Although a more complex protocol, it is nonetheless highly efficient and many aspects of the study can be applied to Mu-DNA. For example, fine tuning of pH and phosphate concentration for lysis of specific sample types could lead to increased DNA yields. Our method uses chemical flocculation of inhibitors from extracted DNA and is pH sensitive (see Dong et al. 2006). For this reason, we did not explore the higher pH lysis of Lever et al. (2015) and it remains an aspect open for future investigation.

Fish metabarcoding of lake water DNA extractions

After the application of noise filtering thresholds to read count data, both methods detected the same 15 fish species previously recorded in Windermere (Hänfling et al. 2016). Broadly, individually sequenced samples cluster by site when visualised with NMDS ordination with some variance between replicates (Supplementary file 2). Although species detected varied between method replicates per site (Supplementary file 3), there was no significant difference between methods in overall species relative abundance (ANOSIM: $R = -0.02$, $p = 0.93$) and both methods produced high similarity species profiles for the lake as a whole (Figure 5). This shows that Mu-DNA produces DNA of sufficient quality for metabarcoding approaches even when target DNA concentration is low and that no bias is introduced through the choice of extraction method.

Costing of extraction methods

Mu-DNA protocols cost less per extraction than the commercial kits to which they were compared (Table 2). Initial consumable costs for our method are higher than purchasing a single commercial kit yet the number of extractions covered by this cost is considerable (see Supplementary file 4). For the cost comparison, institutional discounts were not considered. Were they to have been taken into account, the cost of Mu-DNA would be appreciably lower. Lower costs, combined with a modular application across multiple sample types, makes the method an attractive alternative to commercial kits.

Conclusion

The DNA extraction method presented here, Mu-DNA, achieved high purity DNA yields suitable for PCR and other downstream applications. Mu-DNA is an exploration of the concept of a rapid, modular approach to DNA

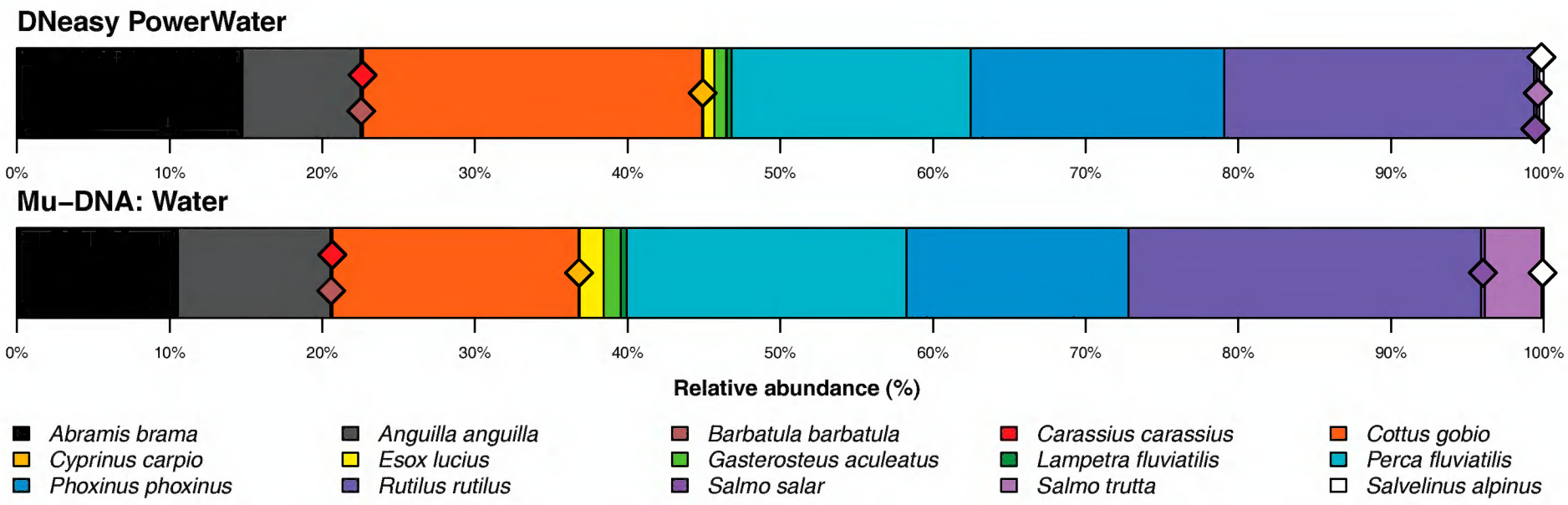

Figure 5. Species profiles of Windermere from metabarcoding of extractions using the compared methods of this study. Relative species abundance (%) of assigned reads is given per method; DNeasy PowerWater or Mu-DNA: Water. Positioning of species is arbitrary and arranged alphabetically. Diamonds indicate the position of low abundance species in the profiles for both methods.

Table 2. Cost per extraction for Mu-DNA protocols and the commercial kits compared in this study.

	Cost per extraction (GBP)
DNeasy PowerSoil (100)	5.24
Mu-DNA: Soil	0.71
DNeasy Blood and Tissue (250)	2.92
Mu-DNA: Tissue	0.67
DNeasy PowerWater (100)	7.03
Mu-DNA: Water	0.83

extraction from a wide range of sample types. Our modular approach to DNA extraction performed as well as, if not better than, the commonly used commercial kits even across challenging samples. This modular adaptability has the potential to be applied to any sample, creating a bespoke DNA extraction to achieve the desired results for the user. As a single, cost effective and comparable alternative to multiple commercial kits, the reliable performance of Mu-DNA allows it to reduce the costs and increase the scope of molecular studies and experiments.

Acknowledgements

Many thanks to Dr James Kitson for the inspiration to design Mu-DNA. Dr Dave Lunt for advice on extraction optimisation. Dr Peter Shum for advice on multiple extraction techniques and sample donation. Marco Benucci, Dr Rosetta C. Blackman, Dr Robert K. Donnelly, Lynsey Harper and Jianlong Li for sample donation and participation in initial protocol optimisation and comparisons. Robert Jaques, Alan Smith and Vic Sweetez for sample collection. Dr Michael A. Braim for sampling permission. Dr Hayley Watson for assistance in field water sample collection. Dr Lori Lawson Handley for feedback on manuscript drafts. Dr James Gilbert for R advice and proofreading. Lisa Malm for adopting our method and the feedback given. Dr Ian Winfield and the Windermere FBA site for laboratory facilities during fieldwork.

References

Aagaard K, Petrosino J, Keitel W, Watson M, Katancik J, Garcia N, Patel S, Cutting M, Madden T, Hamilton H, Harris E, Gevers D, Simone G, McInnes P, Versalovic J (2013) The Human Microbiome Project strategy for comprehensive sampling of the human microbiome and why it matters. FASEB Journall: official publication of the Federation of American Societies for Experimental Biology 27: 1012–1022. https://doi.org/10.1096/fj.12-220806

Athanasio CG, Chipman JK, Viant MR, Mirbahai L (2016) Optimisation of DNA extraction from the crustacean *Daphnia*. PeerJ 4: e2004. https://doi.org/10.7717/peerj.2004

Bolnick DI, Snowberg LK, Hirsch PE, Lauber CL, Knight R, Caporaso JG, Svanbäck R (2014) Individuals' diet diversity influences gut microbial diversity in two freshwater fish (threespine stickleback and Eurasian perch). Ecology Letters 17: 979–987. https://doi.org/10.1111/ele.12301

Braid MD, Daniels LM, Kitts CL (2003) Removal of PCR inhibitors from soil DNA by chemical flocculation. Journal of Microbiological Methods 52: 389–393. https://doi.org/10.1016/S0167-7012(02)00210-5

Brolaski MN, Venugopal RJ, Stolow D (2008) Kits and processes for removing contaminants from nucleic acids in environmental and biological samples. US Patent.

Bürgmann H, Pesaro M, Widmer F, Zeyer J (2001) A strategy for optimizing quality and quantity of DNA extracted from soil. Journal of Microbiological Methods 45: 7–20. https://doi.org/10.1016/S0167-7012(01)00213-5

Colston TJ, Noonan BP, Jackson CR (2015) Phylogenetic Analysis of Bacterial Communities in Different Regions of the Gastrointestinal Tract of *Agkistrodon piscivorus*, the Cottonmouth Snake. PloS One 10: e0128793. https://doi.org/10.1371/journal.pone.0128793

Crouse J, Amorese D (1987) Ethanol precipitation: ammonium acetate as an alternative to sodium acetate. Focus 9: 3–5.

Davis TE, Grothe AM, Schwartz HL, Gripp J, Morrow DG, Van Huystee S (1997) DNA isolating apparatus comprising a non-porous DNA binding, anion exchange resin and methods of use thereof. US Patent.

Deiner K, Renshaw MA, Li Y, Olds BP, Lodge DM, Pfrender ME (2017) Long-range PCR allows sequencing of mitochondrial genomes from environmental DNA. Methods in Ecology and Evolution / British

Ecological Society 8: 1888–1898. https://doi.org/10.1111/2041-210X.12836

Dong D, Yan A, Liu H, Zhang X, Xu Y (2006) Removal of humic substances from soil DNA using aluminium sulfate. Journal of Microbiological Methods 66: 217–222. https://doi.org/10.1016/j.mimet.2005.11.010

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R (1994) DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3: 294–299.

Hänfling B, Lawson Handley L, Read DS, Hahn C, Li J, Nichols P, Blackman RC, Oliver A, Winfield IJ (2016) Environmental DNA metabarcoding of lake fish communities reflects long-term data from established survey methods. Molecular Ecology 25: 3101–3119. https://doi.org/10.1111/mec.13660

Ishaq SL, Wright A-D (2014) High-throughput DNA sequencing of the ruminal bacteria from moose (*Alces alces*) in Vermont, Alaska, and Norway. Microbial Ecology 68: 185–195. https://doi.org/10.1007/s00248-014-0399-0

Kelly RP, Port JA, Yamahara KM, Crowder LB (2014) Using environmental DNA to census marine fishes in a large mesocosm. PloS One 9: e86175. https://doi.org/10.1371/journal.pone.0086175

Kitson JJN, Hahn C, Sands RJ, Straw NA, Evans DM, Lunt DH (2018) Detecting host-parasitoid interactions in an invasive Lepidopteran using nested tagging DNA-metabarcoding. Molecular Ecology. https://doi.org/10.1111/mec.14518

Klingstrom T, Bongcam-Rudloff E, Pettersson OV (2018) A comprehensive model of DNA fragmentation for the preservation of High Molecular Weight DNA. bioRxiv: 254276. https://doi.org/10.1101/254276

Knapp BA, Seeber J, Rief A, Meyer E, Insam H (2010) Bacterial community composition of the gut microbiota of *Cylindroiulus fulviceps* (diplopoda) as revealed by molecular fingerprinting and cloning. Folia Microbiologica 55: 489–496. https://doi.org/10.1007/s12223-010-0081-y

Koinari M, Karl S, Ng-Hublin J, Lymbery AJ, Ryan UM (2013) Identification of novel and zoonotic *Cryptosporidium* species in fish from Papua New Guinea. Veterinary Parasitology 198: 1–9. https://doi.org/10.1016/j.vetpar.2013.08.031

Lau YL, Chang PY, Subramaniam V, Ng YH, Mahmud R, Ahmad AF, Fong MY (2013) Genetic assemblage of *Sarcocystis* spp. in Malaysian snakes. Parasites & Vectors 6: 257. https://doi.org/10.1186/1756-3305-6-257

Lever MA, Torti A, Eickenbusch P, Michaud AB, Šantl-Temkiv T, Jørgensen BB (2015) A modular method for the extraction of DNA and RNA, and the separation of DNA pools from diverse environmental sample types. Frontiers in Microbiology 6: 476. https://doi.org/10.3389/fmicb.2015.00476

Lewis WB, Moore FR, Wang S (2016) Characterization of the gut microbiota of migratory passerines during stopover along the northern coast of the Gulf of Mexico. Journal of Avian Biology 47: 659–668. https://doi.org/10.1111/jav.00954

Lloyd DR, Phillips DH (1999) Oxidative DNA damage mediated by copper (II), iron (II) and nickel (II) Fenton reactions: evidence for site-specific mechanisms in the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intrastrand cross-links. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 424: 23–36. https://doi.org/10.1016/S0027-5107(99)00005-6

Mayjonade B, Gouzy J, Donnadieu C, Pouilly N, Marande W, Callot C, Langlade N, Muños S (2016) Extraction of high-molecular-weight genomic DNA for long-read sequencing of single molecules. BioTechniques 61: 203–205. https://doi.org/10.2144/000114460

Niemiller ML, Porter ML, Keany J, Gilbert H, Fong DW, Culver DC, Hobson CS, Denise Kendall K, Davis MA, Taylor SJ (2017) Evaluation of eDNA for groundwater invertebrate detection and monitoring: a case study with endangered *Stygobromus* (Amphipoda: Crangonyctidae). Conservation Genetics Resources: 1–11. https://doi.org/10.1007/s12686-017-0785-2

Ogram A, Sayler GS, Barkay T (1987) The extraction and purification of microbial DNA from sediments. Journal of Microbiological Methods 7: 57–66. https://doi.org/10.1016/0167-7012(87)90025-X

Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H (2017) vegan: Community Ecology Package. R package version 2.4-3. https: //CRAN.R-project.org/package=vegan

Olson ND, Morrow JB (2012) DNA extract characterization process for microbial detection methods development and validation. BMC Research Notes 5: 668. https://doi.org/10.1186/1756-0500-5-668

O'Rorke R, Cobian GM, Holland BS, Price MR, Costello V, Amend AS (2015) Dining local: the microbial diet of a snail that grazes microbial communities is geographically structured. Environmental Microbiology 17: 1753–1764. https://doi.org/10.1111/1462-2920.12630

Parfrey LW, Walters WA, Lauber CL, Clemente JC, Berg-Lyons D, Teiling C, Kodira C, Mohiuddin M, Brunelle J, Driscoll M, Fierer N, Gilbert JA, Knight R (2014) Communities of microbial eukaryotes in the mammalian gut within the context of environmental eukaryotic diversity. Frontiers in Microbiology 5: 298. https://doi.org/10.3389/fmicb.2014.00298

Pitcher DG, Saunders NA, Owen RJ (1989) Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. Letters in Applied Microbiology 8: 151–156. https://doi.org/10.1111/j.1472-765X.1989.tb00262.x

R Core Team (2016) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https: //www.R-project.org/

Rees HC, Maddison BC, Middleditch DJ, Patmore JRM, Gough KC (2014) REVIEW: The detection of aquatic animal species using environmental DNA – a review of eDNA as a survey tool in ecology. The Journal of Applied Ecology 51: 1450–1459. https://doi.org/10.1111/1365-2664.12306

Riaz T, Shehzad W, Viari A, Pompanon F, Taberlet P, Coissac E (2011) ecoPrimers: inference of new DNA barcode markers from whole genome sequence analysis. Nucleic Acids Research 39: e145. https://doi.org/10.1093/nar/gkr732

Robe P, Nalin R, Capellano C, Vogel TM, Simonet P (2003) Extraction of DNA from soil. European Journal of Soil Biology 39: 183–190. https://doi.org/10.1016/S1164-5563(03)00033-5

Rohland N, Reich D (2012) Cost-effective, high-throughput DNA sequencing libraries for multiplexed target capture. Genome Research 22: 939–946. https://doi.org/10.1101/gr.128124.111

Singh R, Gupta V, Verma V, Rasool S (2014) A modified and improved method for isolation of metagenomic DNA from hilly and dense forest soil samples. International Journal of Advanced Technology in Engineering and Science 2: 545–557.

Spens J, Evans AR, Halfmaerten D, Knudsen SW, Sengupta ME, Mak SST, Sigsgaard EE, Hellström M (2016) Comparison of capture and storage methods for aqueous macrobial eDNA using an optimized extraction protocol: advantage of enclosed filter. Methods in Ecology and Evolution / British Ecological Society. https://doi.org/10.1111/2041-210X.12683

Tebbe CC, Vahjen W (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. Applied and Environmental Biology 59: 2657–2665.

Vo A-TE, Jedlicka JA (2014) Protocols for metagenomic DNA extraction and Illumina amplicon library preparation for faecal and swab samples. Molecular Ecology Resources 14: 1183–1197. https://doi.org/10.1111/1755-0998.12269

Wechter P, Williamson J, Robertson A, Kluepfel D (2003) A rapid, cost-effective procedure for the extraction of microbial DNA from soil. World Journal of Microbiology and Biotechnology 19: 85–91. https://doi.org/10.1023/A: 1022587806945

Williams JA, Yeggy JP, Markovetz AJ (1980) Role of nucleases in the isolation of plasmid deoxyribonucleic acid from *Pseudomonas cepacia* 4G9. Journal of Bacteriology 143: 1057–1059.

Wilson IG (1997) Inhibition and facilitation of nucleic acid amplification. Applied and Environmental Biology 63: 3741–3751.